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Effects of Germination Conditions of Brown Rice in Relation to Flour Physicochemical Properties and Bread Qualities

Wipada Wunthunyarat
University of Arkansas, Fayetteville

Effects of Germination Conditions of Brown Rice in Relation to Flour Physicochemical Properties and Bread Qualities

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Wipada Wunthunyarat
Kasetsart University
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_____
Ya-Jane Wang, Ph.D.
Thesis Director

_____
Andy Mauromoustakos, Ph.D.
Committee Member

_____
Han-Seok Seo, Ph.D.
Committee Member

Abstract

Gluten-free products from rice are gaining popularity among consumers because of its hypoallergenic characteristic. The absence of gluten results in inferior bread qualities such as hard texture, reduced volume and shorter shelf-life, which can be improved by the addition of external hydrolytic enzymes. Hydrolytic enzymes are activated during germination to stimulate plant growth, and hence these enzymes may function similarly to the external enzymes to improve gluten-free bread from brown rice. Therefore, the objective of this work was to investigate the activities of amylases and protease in germinated brown rice (GBR) from long-grain and short-grain rices under different germination conditions and their impacts on flour properties and bread qualities. Brown rice (BR) was germinated aerobically and anaerobically for 2 and 4 days, and then assayed for alpha-amylase, beta-amylase, alpha-glucosidase, and protease activities, chemical composition, physiochemical properties and starch size distribution. GBR from long-grain rices displayed greater enzyme activities, resulting in more changes in chemical composition, physiochemical properties and starch size distribution. Breads were prepared from GBR along with BR (control) of long-grain rice and evaluated for specific volume, texture, retrogradation, color and starch characteristics. The results showed that breads prepared from GBR flour showed a greater specific volume (4-10%), a reduced hardness (34-90%), and a lower starch retrogradation (66-90%) compared with the control. After stored for 5 days, breads prepared from GBR flour exhibited no change in specific volume and less hardness and retrogradation than the control bread, which was supported by the significant reduction of starch molecular size. In conclusion, the hydrolytic enzymes activated from germination significantly improved physicochemical properties of BR flour and consequently bread qualities. Furthermore, rice germinated under aerobic condition for 4 days exhibited better properties.

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I. Introduction

Germinated brown rice (GBR) is considered as a functional food because germination creates and enhances bioactive compounds, such as γ-aminobutyric acid (GABA), γ-oryzanol, α-tocopherol and phenolic compounds. Germination activates hydrolytic enzymes such as α-amylase, protease and lipase, resulting in the degradation of starch, protein and lipids, respectively. The enzymatic changes during germination improve functional properties of GBR flour, such as lower viscosity, greater water and oil absorption, and improved foaming capacity.

Gluten-free products have attracted attention recently because of their increasing popularity among consumers. Gluten-free diets are not only for celiac patients who are unable to digest gluten, i.e. protein found in wheat, rye and barley, resulting in gastrointestinal tract inflammation, but also for health-conscious people. However, there are challenges in replacing wheat with other flours, such as poor crust and crumb, poor mouth-feel, fast staling and reduced volume. Thus, additives, such as starch, hydrocolloids, milk protein, and transglutaminase, have been added and shown to improve binding and foaming properties of gluten-free products.

Because of the transformation of starch and protein during germination that has been reported to improve water absorption and foaming capacity, it is hypothesized that germinated brown rice will improve gas expansion and reduce starch retrogradation in gluten-free products, and germinated brown rice from different germination conditions will display different extents of gas expansion and starch retrogradation. Therefore, the objectives of this study are to characterize amylases and protease activities in GBR prepared by different germination conditions and to correlate them with the changes of gas expansion and starch retrogradation in gluten-free products.

II. Literature review

Rice (*Oryza sativa* L.) is the most consumed cereal in the world and cultivated in more than 100 countries across the world (Chang, 2003). Paddy is harvested from the field and then dehulled and polished to produce brown and milled rice, respectively. Cooked brown rice has a harder texture than milled rice. However, germination improves texture of brown rice and enhances nutrients. In addition, germination can improve physicochemical properties of flour such as water absorption and foaming capacity.

1. Rice Composition

Rice grain structure consists of hull and caryopsis as shown in Fig. 1. The hull comprises awn, lemma, palea, sterile lemmae and rachilla. The caryopsis consists of pericarp, seed coat, aleurone layer, endosperm and embryo. Milling of rough rice removes the hull, resulting in brown rice with lower proteolytic and lipolytic activities (Zhou, Robards, Helliwell, & Blanchard, 2002). The components of rice typically are affected by cultivars and growing environment. However, they approximate as following: 7.2% protein, 66.4% starch, 26.5% amylose, 0.7% crude fiber, 2.9% crude fat and 0.78% ash (Resurreccion, Juliano, & Tanaka, 1979).

1.1 Starch

Starch is the main component in rice grain endosperm with an irregular polygonal shape (Hayakawa, Seo, & Igaue, 1980) and as compound granules with a size of 3-8 μm, which are considered as the smallest one among all starch-producing grains (Ellis et al., 1998). Starch granules are semi-crystalline granules comprising linear amylose and highly branched amylopectin molecules (Jenkins & Donald, 1995) whose proportion and chain length affect

physicochemical properties of starch, for example, pasting properties (Jane et al., 1999) and gelatinization temperature.

Amylose is composed of linear chains of approximately 200-2000 anhydroglucose units that are linked by α-D-1,4 glucosidic bonds with a reducing end and a non-reducing end. Amylose can form a left-handed single (Fig. 2.) and parallel left-handed double helix, which forms complex with iodine to render a blue color. Intermolecular attraction of amylose occurs in an aqueous solution of amylose, resulting in increasing viscosity and retrogradation.

Amylopectin is a highly branched structure with additional α-D-1,6 glucosidic bonds that occurs every 20-30 anhydroglucose units. Aqueous solution of amylopectin is high in viscosity, clarity, and stability and resistant to gelling. Amylopectin chains are classified as A, B and C chains (Fig. 3.). The A chains are the short branch chains attached to other chains without any chains attached to them. The B chains are the chains attached to others through (1→6) linkages. The B1, B2 and B3 chains are chains across one, two and three clusters, respectively. The C chains are the main chains with a reducing end (Hizukuri, 1986).

Starch granules are structured in clusters of semi-crystalline form with amylopectin chains arranged in alternating crystalline and amorphous lamellae and amylose interspersing among amylopectin chains. There are three types of crystallite found in starch granules of different sources, i.e. A, B and C (Fig. 4.). The A-type is densely packed crystallite in an orthogonal pattern with 8 water molecules in the crystallite, such as cereal starches like rice starch; The B-type is loosely packed in a hexagonal pattern with 36 water molecule, such as tuber and root starches; The C-type is the mixture of A- and B-type, such as bean starches.

Starch granule is insoluble in water because of its semi-crystalline structure. However, elevated temperature can increase its water absorption, resulting in granule swelling. At a certain temperature that the energy overcomes the hydrogen bonding in the crystallites, water uptake becomes irreversible and crystallinity disappears, which is called gelatinization (Miles, Morris, & Ring, 1985). During swelling, starch molecules leach out of granules, mostly amylose and small amylopectin molecules. During cooling and storage, starch molecules re-associate to form crystallites, which are not as ordered as those in their native forms, known as retrogradation (Gudmundsson, 1994). This process affects the texture and shelf-life of starch-containing products such as bread.

1.2 Protein

Rice protein is largely located in endosperm, mainly in the subaleurone layers, (Singh, 1998). The amount of protein depends on the degree of polishing (Pal, Pandey, & Sah, 1999), cultivar and growing environment. Glutelin (alkali-soluble) is the predominant protein fraction of more than 80% in rice, followed by ~10% globulin (salt-soluble), ~5% albumin (water-soluble) and less than 5% prolamin (alcohol-soluble). Protein plays an important role in network formation in gluten-free products. Protein and starch can interact and form structure in dough, lending in ability to retain gas during fermentation (Hoseney, 1986). Furthermore, protein also plays a role in water absorption of flour (Ohm & Chung, 1999).

1.3 Lipids

There are three types of lipids in starch, typically in the form of spherosome or droplet in the aleurone layer and embryo: starch lipids, starch surface lipids and non-starch lipids (Morrison, 1978, 1981). Starch lipids are monoacyl lipids, free fatty acids and lysophospholipids

and are located inside the native starch granules. Starch surface lipids are mostly monoacyl lipids and can form complexes with amylose on the surface of starch granules (Ito, Sato, & Fujino, 1979). Non-starch lipids, which are mostly triglyceride and a small amount of free fatty acids and monoacyl lipids, are found in the bran layer and responsible for oxidation in brown rice after the husk is removed. Starch lipids and starch surface lipids also affect starch properties such as granular swelling, gelatinization, viscosity and retrogradation. Lorenzo, Zaritzky, and Califano (2009) reported that lipids were the most important ingredient affecting dough rheology and overall quality of bakery products. Starch surface lipids enhance the dough stability by acting as a surface active substance, resulting in improved gas retention (Gan, Ellis, & Schofield, 1995; Alvarez-Jubete, Auty, Arendt, & Gallagher, 2010). However, non-starch lipids affect negatively on loaf volume (Gan, Ellis, & Schofield, 1995).

2. Germination Process

Germination is a biotransformation process when a dry seed initiates water uptake and a sprout expands to 2-5 mm (Watanabe et al., 2004). During germination, hydrolytic enzymes, such as α-amylase, protease and lipase, are activated for the plant growth (Cho & Lim, 2016). According to Counce, Keisling, and Mitchell (2000), rice germination can be divided into four stages: an unimbibed seed, the emergence of coleoptile from the seed, the growth of radical, and the emergence of prophyll from coleoptile (Fig. 5). After the growth of prophyll, rice begins the vegetative stage.

Rice can be germinated from rough rice and brown rice (Moongngarm & Saetung, 2010) at 30-35°C (Nishiyama, 1976) and 80-85% humidity for 24-72 h (Capazana & Buckle, 1997) after soaking at 25-30°C for 12-24 h. Germinated rough rice is higher in sugar, free amino acids,

vitamin B and bioactive compounds than GBR because the husk protects the leaching of nutrients (Moongngarm & Saetung, 2010). Charoenthaikij et al. (2009) found that increased soaking time and decreased pH resulted in higher in the amount of reducing sugar, GABA and α-amylase activity but lower in total starch and pasting viscosities. Increased relative humidity and humidification time reduce the time to achieve 50% germination rate (Lee, Kim, Hong, & Yun, 1998). Germination in darkness inhibits strigolactone, a plant growth hormone, resulting in a shorter coleoptile (Hu et al., 2010). Palmiano and Juliano (1972) found that the degradation of starch and protein was faster when germinated in darkness because no photosynthesis takes place.

GBR was first commercialized in Japan in 1995 (Patil & Khan, 2011) and became popular among health-conscious consumers because of its potential health-promoting components (Cho & Lim, 2016), such as phenolic compounds, and γ-aminobutyric acid (GABA) (Moongngarm & Saetung, 2010). GABA is known as a neurotransmitter in central nervous system, which can accelerate metabolism in brain, decrease blood pressure and reduce Alzheimer's disease and oxidative stress.

Rice germination can occur aerobically and anaerobically. Rice is the only crop that can be germinated in the absence of O_2 environment (Perata & Alpi, 1993) because under anaerobic condition, gibberellic acid in rice still can induce the production of α-amylase, resulting in the breakdown of starch into sugars that are an energy source for rice growth, while other crops do not response to gibberellic acid when germinating anaerobically, resulting in the inability to produce α-amylase (Perata, Geshi, Yamaguchi, & Akazawa, 1993). Anaerobic germination is believed to be a biofortification process since it increases a larger amount of nutrients such as GABA, lysine and D-mannose than aerobic condition (Ding et al., 2016).

2.1 Enzyme activity changes during germination

Hydrolytic enzymes are activated at the beginning of germination. Alpha-amylase is mostly located in the aleurone layer and is responsible for starch degradation into sugars, such as glucose, maltose and maltotriose (Murata, Akazawa, & Fukuchi, 1968). The maximum amounts of glucose and maltose were found at the third day of germination but these levels decreased afterward because the sugars were used for the formation of roots and shoots (Saman, VáZquez, & Pandiella, 2008). The synthesis of α-amylase is induced by gibberellic acid (Tanaka, Ito, & Akazawa, 1970; Murata, Akazawa, & Fukuchi, 1986; Chrispeels & Varner, 1966), a plant growth hormone located in the embryo area. Alpha-amylase hydrolyzes starch randomly from non-reducing ends and slows down and stops near α-D-(1→6) linkage (Briggs, Hough, Stevens, & Young, 1981). Shu, Frank, Shu, and Engel (2008) found the degradation of starch by 43% and the increase in reducing sugar by 40-fold after germinating for 7 days. Germination in dark conditions results in more rapid starch degradation than in the presence of light (Palmiano & Juliano, 1971). The activity of α-amylase is affected by the amylose/amylopectin ratio (Saman, VáZquez, & Pandiella 2008). Palmiano and Juliano (1971) discovered the α-amylase isozyme during the germination process, which indicates the formation of α-amylase. Beta-amylase is also activated during germination. The initial activity of β-amylase is greater than that of α-amylase but after 7 days of germination, β-amylase activity is lower than α-amylase activity (Palmiano & Juliano, 1971). Another amylolytic enzyme activated during germination is α-glucosidase that hydrolyzes starch from the reducing end and can hydrolyze both α-(1→4) and α-(1→6) linkages, producing glucose (Saman, VáZquez, & Pandiella 2008).

Protease is responsible for the breakdown of protein, primarily located in the aleurone layer and induced by gibberellic acid (Jacobsen & Varner, 1967). Protease activity increases

from the 2nd or 3rd day of germination (Saman, VáZquez, & Pandiella, 2008) and reaches its highest amount at the 5th or 6th day of germination (Palmiano & Juliano, 1971). The products of protein degradation are soluble protein (a main product) and free amino acids that are detected since the first day of germination.

2.2 Chemical changes during germination

Hydrolytic enzymes such as amylolytic enzymes, protease, lipase and phytase are activated during germination because of the presence of water and result in nutrient changes. Crude protein and total free amino acid increase after germination (Moongngarm & Saetung, 2010; Chinma, Anuonye, & Simon, 2015). Crude protein increases because some non-protein nitrogen compounds, such as nucleic acid, are produced; total free amino acid increases because of the degradation of protein into amino acids by protease. However, total starch, amylose and amylopectin decrease because of hydrolysis of starch by amylolytic enzymes during germination (Chinma, Anuonye, & Simon, 2015; Wu et al., 2013).

Physicochemical properties of rice flour change during germination, for example, water and oil absorption, foaming capacity and stability and pasting properties. Water and oil absorption and foaming capacity improve because when protein is hydrolyzed by protease, both hydrophilic and hydrophobic sites are exposed and become available to hold more water and oil, respectively. When air bubbles are created, the hydrophobic sites will face the air phase, while hydrophilic sites will face the water phase, thus stabilizing the air bubbles. Pasting viscosities decrease because the activated α-amylase hydrolyzes starch during the heating process, as evidenced by the lack of change in viscosity when 0.50 mM silver nitrate is used instead of water, which inhibits α-amylase activity (Han et al., 2016).

3. Gluten-free Bread

Celiac disorder is an autoimmune gastrointestinal tract disorder where the digestion of gluten (protein found in wheat, rye and barley) produces hydrolyzed protein such as peptides relating to an immune response that damages the villi in the small intestine, resulting in inability to absorb nutrients (Turkut, Cakmak, Kumcuoglu, & Tavman, 2016; Tye-Din & Anderson, 2008). Gluten-free products are popular among celiac patients; however, there are challenges to substitute wheat flour with other flours. The absence of gluten results in poor crumb and crust, poor mouth-feel, liquid dough, fast staling (Moroni, Bello, & Arendt, 2009) and reduced volume of bread due to insufficient gas expansion and retention (Mariotti, Lucisano, Pagani, & Ng, 2009). Thus, additives such as hydrocolloids, milk protein, transglutaminase, protease, α-amylase and α-glucosidase have been used to improve gluten-free bread qualities.

Hydrocolloids have been added in gluten-free products to improve the viscoelastic properties for stretch ability (Ronda, Perez-Quirce, Lazaridou, & Biliaderis, 2015). Milk protein improves crumb texture and delays the staling by forming a gluten-like matrix in bread (Mariotti, Lucisano, Pagani, & Ng, 2009). Protease increases specific volume, reduces crumb hardness and creates fine bubble cells in crumb because protease hydrolyzes protein into smaller molecules that can form strong network to retain CO_2 during fermentation (Hatta, Matsumoto, & Honda, 2015; Kawamura-Konishi, Shoda, Koga, & Honda, 2013). Elgeti et al. (2014) demonstrated that higher α-glucosidase and α-amylase activities were responsible for the increased volume of gluten-free bread by 25% and 6%, respectively. Both enzymes hydrolyze starch into sugars that are substrates for the growth of yeast.

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5. Figures

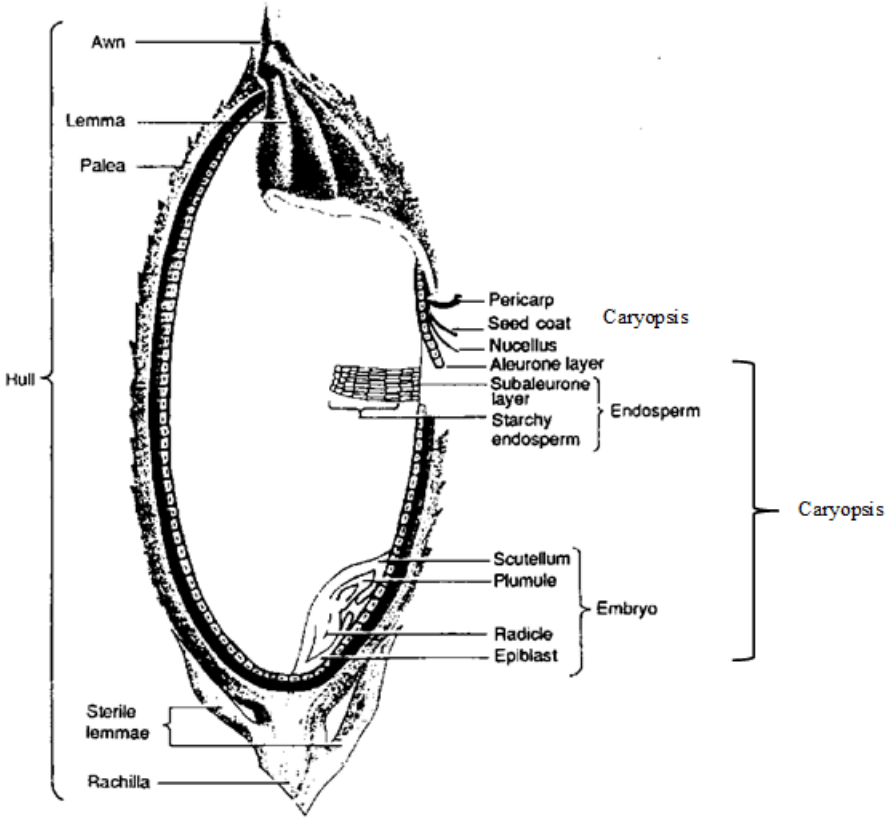


Figure 1 A structure of rice grain (Juliano, 1985).

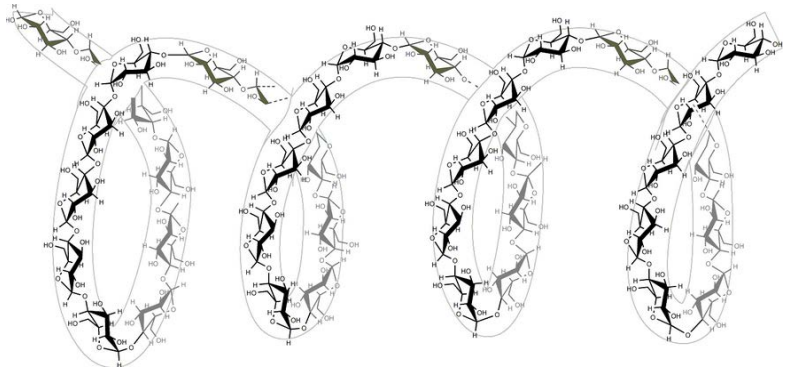


Figure 2 Helical arrangement of amylose molecule. (Reid, 2003)

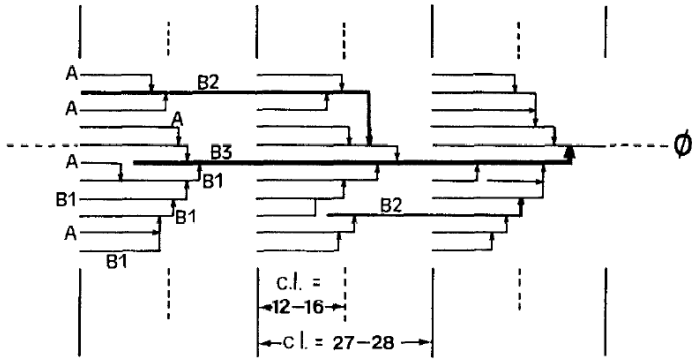


Figure 3 A cluster model for amylopectin (Hizukuri, 1986).

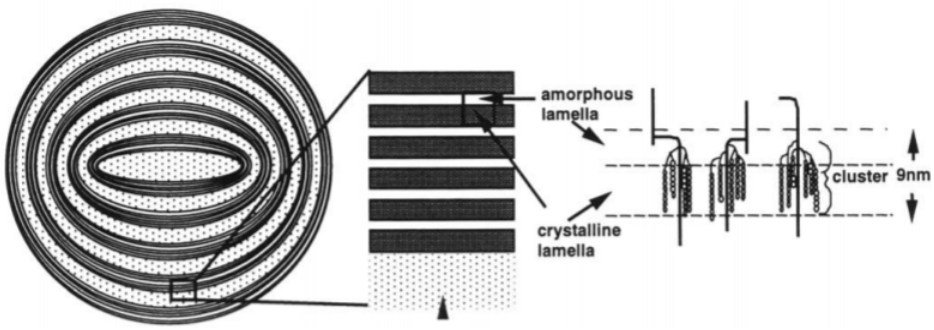


Figure 4 Starch granule with growth ring and amorphous and crystalline lamellae (Donald et al., 1997; Tester, Karkalas, & Qi, 2004).

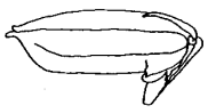
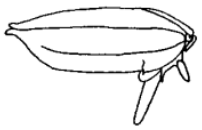
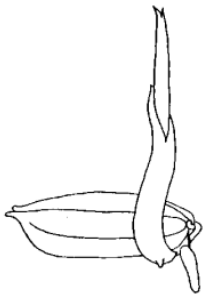
Growth Stage	S0	S1	S2	S3
Morphological Criteria	Dry, unimbibed seed	Emergence of coleoptile	Emergence of radicle	Emergence of prophyll from coleoptile
Illustration				

Figure 5 Rice seedling growth stages (Counce, Keisling, & Mitchell, 2000).

III. Effects of Germination Conditions of Brown Rice in Relation to Flour Physicochemical Properties and Bread Qualities

1. Introduction

Gluten-free products from rice are gaining popularity among consumers because of its hypoallergenic property. However, the substitution of wheat flour is challenging because the absence of gluten results in poor crumb and crust characteristics, poor mouth-feel, liquid dough, fast staling (Moroni, Bello, & Arendt, 2009) and reduced volume of bread due to insufficient gas expansion and retention, which affect product's shelf-life and consumer acceptance (Mariotti, Lucisano, Pagani, & Ng, 2009). Recently, bacterial hydrolytic enzymes, such as α-amylase, α-glucosidase and protease, have been used to improve volume and texture of gluten-free products. Sugars from hydrolyzed starch by amylolytic enzymes promote the growth of yeast. Hydrolyzed protein by protease forms a strong structure to retain CO_2 during fermentation, thus increasing specific volume, reducing crumb hardness and creating fine air cells in bread crumb (Hatta, Matsumoto, & Honda, 2015; Kawamura-Konishi, Shoda, Koga, & Honda, 2013). Elgeti et al. (2014) found that 4.5 U/g of α-glucosidase and 0.025 U/g of α-amylase from fungi were responsible for the increased volume of gluten-free bread by 25% and 6%, respectively.

Germination has been employed to improve cooked brown rice texture and enhance potential health-promoting properties by inducing the formation of bioactive compounds, such as gamma-aminobutyric acid (GABA), a neurotransmitter in central nervous system. Germination is a biotransformation process that activates hydrolytic enzymes to provide nutrients for plant growth. These enzymes are induced by a plant hormone, gibberellic acid, in the aleurone layer. Rice is the only cereal that can germinate and grow under either aerobic or anaerobic conditions.

In the absence of oxygen, rice can still produce hydrolytic enzymes because of its ability to respond to gibberellic acid, while the other cereals such as wheat and barley are unable to. It has been reported that germinated rices prepared under different germination conditions contain different enzyme activities that would change the physiochemical properties of the resultant flours. Palmiano and Juliano (1972) reported that a faster starch degradation was observed when rice was germinated in the dark than in the presence of light. The activities of amylases, e.g. α-amylase, β-amylase and α-glucosidase, were greater in rice germinated aerobically than anaerobically (Guglielminetti, Yamaguchi, Perata, & Alpi, 1995). The chemical composition of germinated rice is also affected by hydrolytic enzymes. After germination, crude protein and free amino acid contents increase because of the production of nucleic acid and the degradation of protein by protease, respectively (Chinma et al., 2015; Moongngarm & Saetung, 2010). In contrast, starch and amylose contents decrease after germination because of the degradation of starch by amylolytic enzymes (Chinma et al., 2015; Wu et al., 2013). Furthermore, rice flour properties change after germination, such as foaming capacity, water and oil absorption, and pasting properties. Water and oil absorption and foaming capacity increase because more hydrophilic and hydrophobic sites of protein are exposed after being hydrolyzed by protease, and thus more water, oil and air can be retained. Pasting viscosities decrease from the activation of endo-acting α-amylase that hydrolyzes starch, as supported by an increase in viscosity with the addition of silver nitrate that inactivates α-amylase (Batey, Curtin, & Moore, 1997; Han et al., 2016).

Germinated brown rice (GBR) has been evaluated in gluten-free bread for its increase in protein and starch digestibility, nutritive value and bioactive compounds (Cornejo et al., 2015). Recently, Cornejo and Rosell (2015) studied the effect of germination time on the qualities of

brown rice bread and found that brown rice germinated for 24 h produced breads with an improvement in texture. However, there is little research on how hydrolytic enzyme activities affect dough rheology and starch hydrolysis in relation to gluten-free bread qualities. Therefore, the objectives of this study were to determine the activities of hydrolytic enzymes, including α-amylase, β-amylase, α-glucosidase and protease, and to characterize the physiochemical properties of GBR of varying amylose contents prepared under different germination conditions in relation to dough and bread properties.

2. Materials and Methods

2.1 Materials

Two rice cultivars with different amylose contents were used in this study. Rough rice of a long-grain cultivar, RoyJ, and a short-grain cultivar, RU9601099, from the 2016 crop were provided by the University of Arkansas Rice Research and Extension Center (Stuttgart, AR). Rough rice was dehulled three times by Satake dehusker (THU-35, Satake Corp., Hiroshima, Japan) to obtain brown rice. Broken brown rice was removed by Cater-Day sizer (E8956, Carter-Day, MN, USA), and the head brown rice samples were stored at 4°C for further process and analysis.

2.2 Germination process

Three hundred grams of brown rice was soaked in 1.25% NaClO for 30 min for disinfecting (Yang, Basu, & Ooraikul, 2001). Afterward, brown rice was rinsed with deionized water for three times, soaked in excess deionized water overnight, and then rinsed with deionized water. For aerobic germination, steeped brown rice was placed on top of two layers of cotton

cloth, then filled with 700 mL of deionized water, and water was added every 6-12 h. For anaerobic germination, steeped brown rice was placed in a stainless steel tray (23×33×5 cm) with 2,000 mL of deionized water, and water was changed every 6-12 h. The trays were placed in an incubator (APT.line BF, Binder, Tuttlingen, Germany) at 30 °C and 85% humidity for 2 or 4 days. After germination, GBR was washed with deionized water and then dried at 40 °C in a forced draft oven overnight. GBR was ground into flour using an UDY cyclone sample mill (UDY, Ft. Collins, CO, USA) and stored at 4 °C until use.

2.3 Chemical composition

Crude protein was analyzed by a micro-Kjeldahl method according to Approved Method 46-13 (AACC, 2000) using the conversion factor of 5.95 to convert nitrogen content to crude protein content. Crude fat was measured according to Approved Method 30-20 (AACC, 2000) using a Soxhlet apparatus and petroleum ether as the solvent. Ash content was determined according to Approved Method 08–01 (AACC, 2000) by ashing approximately 2 g of ground rice flour at 550 °C for 18 h. The apparent amylose content was measured by iodine colorimetry (Juliano, 1971).

2.4 Flour properties

2.4.1 Water absorption

Water absorption (g/g) was measured according to Approved Method 56-20 (AACC, 2000) with slight modification. One gram of GBR flour was suspended in 10 mL deionized water at room temperature for 30 min, and then centrifuged at 4,000 ×g for 25 min. The supernatant was decanted, and the water absorption was calculated by the ratio of the sediment weight over the flour initial weight.

$$\text{Water absorption (g/g)} = \frac{\text{Weight of sediment (g)}}{\text{Initial weight of flour (g)}}$$

2.4.2 Foaming capacity

Foaming capacity (%) was determined by using a standard electric blender (Waring commercial 51BL31, CT, USA) at a high speed (No. 2) for blending 2 g of GBR flour with 100 mL of distilled water for 6 min, and then immediately poured the mixture into a 50-mL graduated cylinder. The volumes before and after blending were recorded. Foaming capacity was the ratio of the difference of volume before and after blending over the volume before blending (Elkhlifa & Bernhardt, 2010).

$$\text{Foaming Capacity (\%)} = \frac{\text{Volume after blending} - \text{ volume before blending}}{\text{volume before blending}} \times 100$$

2.4.3 Gelatinization properties

Gelatinization properties were measured using a differential scanning calorimeter (DSC, model Diamond, Perkin-Elmer Co., Norwalk, CT, USA). Approximately 3 mg of rice flour was weighed into an aluminum DSC pan with 6 μL of deionized water added. The pan was sealed and equilibrated at room temperature for an hour prior to heating from 25 °C to 120 °C at 10 °C/min. An empty aluminum DSC pan was used as a reference. Onset, peak, and end temperatures and enthalpy were calculated.

2.4.4 Pasting properties

Pasting properties was characterized with a Rapid ViscoAnalyser (RVA, Newport Scientific Pty. Ltd, Warriewood NSW, Australia). Rice slurry was prepared by mixing 3.0 g of rice flour with 25.0 mL of deionized water, 0.50 mM silver nitrate, 0.1% N-ethylmaleimide, or a mixture of 1.0 mM silver nitrate and 0.2% N-ethylmaleimide, rapidly heated to 50 °C, heated

from 50 °C to 95 °C at 5 °C/min, held at 95 °C for 9 min, and then cooled to 50 °C at 5 °C/min. Peak, breakdown, setback, and final viscosities in centipoise (Cp) were recorded.

2.4.5 Enzymes activities

The activities of α-amylase, β-amylase and α-glucosidase were assayed by using the enzyme extraction and assay protocol for wheat and barley flours of the Ceralpha Method for the measurement of plant and microbial α-amylase by Megazyme (Wicklow, Ireland).

Protease was extracted by following the method of Elkhalifa and Bernhardt (2010). Two grams of the GBR flour was suspended in 10 mL of distilled water at room temperature for 1 h with a rotary shaker and then centrifuged at 4000 ×g for 10 min. The supernatant was collected and analyzed for protease activity using the Pierce Protease Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).

2.4.6 Starch structure characterization

Starch was isolated from GBR flour using the alkali-steeping method described by Yang, Lai, and Lii (1984) with slight modifications. Five grams of GBR flour were soaked in 35 mL of 0.1% NaOH in a 50-mL centrifuge tube, incubated overnight on a rotary shaker, and then filtered through a 63-µm sieve and centrifuged at 1500 ×g for 10 min. The supernatant was discarded, and the yellowish top layer was removed. Afterward, the sample was washed with DI water and adjusted to pH 6.5 with 0.5 M HCl. Lipid was removed by following the method of Patindol and Wang (2002) with water-saturated 1-butanol overnight. Then the defatted sample was centrifuged at 1200 ×g for 10 min, the residue was dry at 40 °C overnight, and the starch was ground and passed through a 250-µm sieve.

The molecular size distribution of the starch was characterized to reveal the extent of degradation as a result of activities of amylolytic enzymes during germination by high performance size-exclusion chromatography (HPSEC) according to Kasemsuwan et al. (1995) with modifications (Patindol & Wang 2002). For native starch, 20 mg of starch was added into 5 mL of 90% DMSO in a 25-mL screw-cap test tube, boiled for an hour, and stirred at room temperature overnight. Then, approximately 2 mL of the samples were centrifuged at 14,000 ×g for 10 min. For debranched starch, 10 mg of starch was mixed with 3.2 mL of Millipore water, boiled for 30 min, cooled down and added with 0.4 mL of acetate buffer pH 3.5. Afterward, it was incubated with 10 µL of isoamylase at 45 °C for 2 h and then boiled for 15 min, and the buffer was removed with exchange resin IONAC NM-60 H^+/OH^--form, Type I (16-50 Mesh).

The HPSEC system (Waters, Milford, MA) consisted of a 515 HPLC pump with a 200-µL sample loop, an in-line degasser, a 2410 refractive index detector maintained at 40 °C, and a series Shodex OHpak columns (KB-802 and KB-804) maintained at 55 °C. Amylopectin, intermediate material, and amylose content were calculated from the area of their corresponding peaks by Empower Software.

2.5 Bread preparation

Only germinated long-grain brown rice was used to prepare gluten-free bread. Bread was prepared following the formulation of Elgeti et al. (2014) with modifications as presented in Table 1. Dry ingredients were mixed manually for 3 min, water was added, and then the dough was manually kneaded for 3 min. Three hundred and fifty grams of dough was placed into a 6.5×13.0×5.5 cm baking pan, fermented at 30 °C and 80-90% humidity for 30 min in an incubator, and baked at 225 °C for 35 min. Afterward, bread was cooled down for an hour before analyses or stored in a polyethylene bag at room temperature for 5 days prior to analyses.

2.6 Dough rheology

Dough rheology was performed using a controlled stress and strain rheometer (TA Instrument AR 2000 EX Rheometer, New Castle, DE, USA) according to the method by Renzetti and Arendt (2009). A parallel plate geometry (50-mm diameter) was used with a gap of 1 mm. Dough was prepared using 5 g of flour and 4 g of water, mixed and incubated in a water bath at 30 °C for 30 min. Five grams of the dough was used for frequency sweeps from 0.1 to 10 Hz with a target strain of 0.1% at a constant temperature of 30 °C. The flow behavior of the dough was also analyzed with shear measurements over the range of 0.06-5.0 s^{-1} as described by Renzetti and Arendt (2009).

2.7 Bread properties

2.7.1 Specific volume

Breads at day 0 (after cooling at room temperature for 1 h) and day 5 were measured for volume by rapeseed displacement according to Approved Method 10-05 (AACC, 2000), and specific volume was calculated as the ratio of volume and weight of bread.

$$\text{Specific volume} = \frac{\text{Volume (cm}^3\text{)}}{\text{Weight (g)}}$$

2.7.2 Texture analysis

Breads at day 0 and day 5 were analyzed for texture by following the method of Elgeti et al. (2014) using a texture analyzer (TA-XT2 Plus, Texture Technologies, Scasrsdale, NY, USA) equipped with a 20-mm aluminum cylindrical probe. Each bread loaf was sliced transversely to obtain slices of 15 mm thickness. Texture profile analysis (TPA) was used to analyze the texture attributes, including hardness, springiness and chewiness, of bread crumb with the conditions of

a compression of 40%, 15 s intermediate rest, downward speed of 1.7 mm/s, upward speed of 1.0 mm/s and a trigger force of 0.05 N.

2.7.3 Starch retrogradation and molecular size distribution

Bread was measured for retrogradation at day 0 and day 5. Bread crumb was dried at 40°C for 20 min, ground and passed through a 250-μm sieve. Approximately 8 mg of ground bread was weighed into a stainless-steel DSC pan with 16 μL of deionized water added and analyzed as described in 2.4.3. The obtained enthalpy value was used to represent as retrogradation.

Starch was extracted from 100 mg of ground bread (through a 250-μm sieve) with 5 mL 90% dimethyl sulfoxide (DMSO) in a boiling water bath for 1 h. Afterward, the mixture was centrifuged at 4,000 ×g for 10 min, and then 4 mL of the supernatant was vortexed with 20 mL pure ethanol for 1 min and centrifuged at 4,000 ×g for 10 min. The supernatant was discarded, 5 mL acetonitrile was slowly added to the precipitate, and the mixture was boiled for 5 min and centrifuged at 4,000 ×g for 10 min. The residue was recovered and dried at room temperature overnight prior to starch structure analysis as described in *2.4.6*.

2.7.4 Crust color

Bread crust color was measured by using a colorimeter (Chroma Meter CR-300, Konica Minolta, Japan) using CIE L*, a*, b* system.

2.8 Statistical analysis

A three-way analysis of variance (ANOVA), treating rice cultivar (long-grain rice versus short-grain rice), germination condition (aerobic versus anaerobic), and germination duration (2 versus 4 days) as main effects, along with their interactions, were conducted using JMP Pro

software (version 14.1, SAS Software Institute, Cary, NC, USA). If a significant difference in means was indicated by the ANOVA, post hoc comparisons were performed using Student's *t*-test ($\alpha = 0.05$).

3. Results and Discussion

3.1 Flour properties

3.1.1 Chemical composition and gelatinization and functional properties

The chemical compositions and gelatinization and functional properties of both long-grain and short-grain brown rices and their germinated counterparts are presented in Table 2. The protein content in both rices increased with increasing germination duration and under aerobic condition, which was attributed to the activation of enzymes and the production of hydrolyzed proteins and non-protein nitrogen substances, e.g. nucleic acids, as proposed by Chinma et al. (2015) and Moongngarm and Saetung (2010). Both lipid and apparent amylose of both rices decreased with increasing germination time and under aerobic condition, because they were degraded to provide energy for biochemical and physicochemical changes during germination (Chinma et al., 2015). The ash content in both rices decreased; however, a more significant decrease was found in anaerobic condition because of solubilization of ash by the soaking water used in anaerobic condition. The sugar content was greater with increasing germination time and under aerobic condition. The sugar content in short-grain rices was about twice as high as that in long-grain rices under the same germination condition, which was explained by the greater proportion of amylopectin with abundant branches for amylases to act in short-grain rices (Saman, Va´zquez, & Pandiella, 2008).

Gelatinization temperatures, including onset (T_o), peak (T_p) and end (T_e) temperatures, increased, but gelatinization enthalpy decreased with increasing germination time for both rices. The increase in gelatinization temperatures was proposed to be a combination of an increase in sugar content (Wu et al., 2013) and the hydrolysis of the amorphous lamellae as a consequence of amylase actions. The decrease in gelatinization temperature range was due to the hydrolysis of the amorphous lamellae. The reduced enthalpy was attributed to a reduction in starch content (Wu et al., 2013) and the development of loose and cracked surface in starch granules (Moongngarm, 2010), thus resulting in less energy required.

Before germination, short-grain rice having a lower apparent amylose content exhibited a greater water absorption than long-grain rice, suggesting that amylopectin may play an important role in water absorption. Water absorption was significantly increased for both rice cultivars after germination, which was attributed to more ionizable polar amino acids and sugar content that are capable for binding water (Chinma et al., 2015; Elkhalifa, Abd, & Bernhardt, 2010; Vioque et al., 2000). The increase in water absorption was more pronounced for long-grain GBR, and flours of 2-day GBR had a higher water absorption than those of 4-day GBR. The more extensively hydrolyzed protein may lose its water absorption ability in 4-day GBR flours.

Foaming capacity of rice flour is predominantly influenced by its protein content and composition, and increased with increasing germination, as a result of an increase in soluble protein (Chinma et al., 2015). Germinated long-grain rices showed a higher foaming capacity than short-grain rice, which was proposed to due to because of its greater protease activity as discussed later. The greater water absorption and foaming capacity of rice flour after germination is recommended for applications in gluten-free products (Moongngarm, Moontree, Deedpinrum, & Padtong, 2014).

In general, the changes in chemical compositions, gelatinization properties and functional properties were lesser in rices germinated under anaerobic than aerobic condition, likely a result of delayed enzyme production from insufficient ATP in the absence of oxygen (Guglielminetti, Yamaguchi, Perata, & Alpi, 1995). The changes were ascribed to the extent of hydrolysis of starch and protein.

3.1.2 Enzyme activities

The activities of amylolytic enzymes and protease from long-grain and short-grain brown rices germinated under different conditions are depicted in Fig. 6. Long-grain rices displayed greater activities for all enzymes than short-grain rices, possibly because long-grain rices greminated faster than short-grain rices (Han et al., 2016). All enzymes displayed greater activities in aerobic condition because of increased production of ATPs (Guglielminetti, Yamaguchi, Perata, & Alpi, 1995). In addition, their activities increased with increasing germination time, presumably due to the longer germination time needs more nutrients and energy. The activities of β-amylase were higher than those of α-amylase in 2-day germinated rices under both conditions but lower than those of α-amylase in 4-day germinated rice. These results agree with the findings of Nandi, Das, and Sen-Mandi (1995) that β-amylase is responsible for initial stage of germination, and Paul, Mukherjee, and Sircar (1970) that α-amylase is a key enzyme for the germination process because the germination process decreased when α-amylase activity declined.

Many factors affect the activities of hydrolytic enzymes in germinated grains, such as types of cereals, steeping and germination conditions, and kilning temperature (Adams & Rinne, 1980; Taylor & Robbins, 1993; Yin, Zhang, Wang, & Chen, 2002). Germinated barley has the highest activities of amylases among other grains, such as rice, wheat and sorghum, because of

its genetic, growing environment and fast water-uptake rate in the steeping process (Usansa et al., 2011). Longer germination time results in higher enzyme activities to provide sufficient nutrients for plant growth (Guglielminetti, Yamaguchi, Perata, & Alpi, 1995). Okungbowa, Obeta, & Ezeogu (2002) reported that steeping sorghum in $Ca(OH)_2$ enhanced the activity of β-amylase at a higher kilning temperature; however, steeping in KOH reduced its activity.

3.1.3 Pasting properties

The pasting profiles of long-grain and short-grain brown rices and their GBR from different germination conditions in different solutions are presented in Figs. 7 and 8, respectively. Silver nitrate and N-ethymaleimide were used to inactivate α-amylase and protease, respectively. After germination, the peak, through and final viscosities of brown rice dramatically decreased when conducted with water (Fig. 7A). Lower viscosities were found in rices germinated under aerobic condition and for 4 days, agreeing with the results of α-amylase activities under different germination conditions (Fig. 6). The peak and trough viscosities of GBR were similar to those of the control when the pasting properties were conducted with silver nitrate (Fig. 7B), indicating that starch was minimally hydrolyzed during germination even though α-amylase activity increased considerably with the progression of germination. The lower final viscosities with the use in silver nitrate was proposed to result from the hydrolysis of amylose and proteins that was not as easy to re-associate and interfered with starch retrogradation, respectively. When N-ethymaleimide, a protease inhibitor, was employed, the reduction in pasting viscosities was not as significant as with water (Fig. 7C), suggesting that protein protected starch granule from hydrolysis by amylases during heating (Derycke et. al, 2005; Hamaker & Griffin, 1993). When silver nitrate and N-ethymaleimide were used together (Fig. 7D), the pasting profiles of germinated rices were more comparable to that of brown rice.

Nevertheless, the final viscosities of GBR flours were slightly lower, confirming the interference of starch retrogradation from hydrolyzed protein and starch.

The pasting profiles of short-grain brown rice and GBR in water (Fig. 8A) and in N-ethymaleimide (Fig. 8C) exhibited a similar trend as those of long-grain rice but higher overall viscosities due to their lower activities of amylases and a greater proportion of amylopectin (Varavinit et al., 2003; Wu et al., 2013). The pasting viscosities of short-grain rices in $AgNO_3$ (Fig. 8B) and the mixture (Fig. 3D) were greater than those in water but not comparable to those of brown rice as in long-grain rices (Fig. 7B and 7D). The results indicate that amylopectin was degraded to a greater extent in short-grain GBR, presumably because of its greater proportion of amylopectin.

3.1.4 Starch molecular size distribution

The molecular size distributions of native and debranched starches of long-grain and short-grain rices are presented in Figs. 9 and 10, respectively, and summarized in Table 3. The peak chain lengths in degree of polymerization (DP in anhydroglucose unit) of each fraction are shown above the profiles. The molecular size distribution of native starch as characterized by HPSEC can be divided into three fractions: Fraction (Fr.) I (amylopectin), Fr. II (intermediate materials) and Fr. III (amylose). The intermediate materials (Fr. II) in long-grain rices had more distinct structural characteristic and existed as a separated group from Fr. I and Fr. III, and was still prominently present during the progression of germination. In contrast, the Fr. II in short-grain rices was more similar to the Fr. I in structure, and not present at a separate and distinct group. During germination, large molecules were hydrolyzed by amylases, and thus Fr. I decreased and Frs. II and III increased. The decrease was more pronounced in long-grain rice, under aerobic conditions and at 4-day germination (Figs. 9A and 10A), which were corroborated

by the activities of amylases previously discussed (Fig. 6). Although 2-day anaerobically GBR from short-grain rice had a similar proportion of Fr. I as its brown rice, its peak chain length was reduced from 17,846 to 15,623. Similarly, 2-day aerobically GBR from short-grain rice had a greater proportion of Fr. III above DP 165, but 4-day aerobically GBR had a greater proportion below DP 165, although their Fr. III values were close. Therefore, it is important to compare not only the proportions of each fraction but also their HPSEC profiles in order to elucidate their differences.

For debranched starches, Fr. I, Fr. II and Fr. III refer to amylose, long- and short-chain amylopectin, respectively (Figs. 9B and 10B). There was no significant difference among the three fractions of brown rice and GBR of long-grain rices (Fig. 9B). In contrast, short-grain rices exhibited different profiles among samples with an increase in Fr. I and Fr. III, but a decrease in Fr. II (Fig 10B). The peak chain lengths of Fr. II and Fr. III were longer in short-grain rice (DP 65 and 33) than in long-grain rice (DP 62 and 27).

3.2 Dough rheology

The viscoelastic properties of doughs prepared from long-grain brown rice (control) and GBR are presented in Fig. 11. Shear stress was found to be in the linear viscoelastic range (LVR) and shear rate independent. The GBR doughs displayed higher shear stress than the control dough over the LVR range of applied shear rate, indicating that all GBR doughs were more resistant to shear stress and deformation than the control dough. The 4-day aerobically germinated dough had the greatest shear stress that was significantly different from the others. In the frequency sweep study, the storage modulus (G') of the control and GBR doughs was frequency dependent; however, the loss tangent ($\tan\delta$) was frequency independent. It is also found that G', an elastic indicator, was greater than the loss modulus (G''), a viscous indicator, at

the frequency range tested for all doughs, implying a more solid behavior of dough. The germination significantly increased the viscous properties ($G^{"}$), supporting the previous results of pasting viscosities and starch molecular size change.

Germination enhanced the shear stress of dough, which improved the resistance of shear stress and deformation. The latter is an important characteristic in gluten-free products because it enables dough to be proved and formed, and subsequently to attain proper gas expansion and retention. The dough prepared from rice flour has been reported to be liquid-like and inextensible (Pongjaruvat et. al, 2014; Renzetti & Arendt, 2009). The present results demonstrated that germination modified the viscoelastic behavior of dough by increasing both $G^{'}$ and $G^{"}$, suggesting a hardening effect on dough (Matos, Sanz, & Rosell, 2014), which improved gas retention in dough during fermentation. In addition, tanδ, a viscoelastic indicator, of GBR dough was greater than the control dough, suggesting a more viscous dough. The increase in shear stress, $G^{'}$, $G^{"}$ and tanδ in GBR doughs were attributed to the activities of protease and amylases that enhanced the protein-protein and protein-starch interactions.

3.3 Bread properties

3.3.1 Specific volume

High specific volume is desired in breads because it indicates proper aeration and porosity. A challenge with gluten-free bread is insufficient gas expansion and retention that lead to a reduced volume. The specific volumes of breads from GBR flours at day 0 and day 5 were measured (Table 3). At day 0, GBR breads showed higher specific volumes than the control bread (1.59 mL^3/g) with an exception of 2-day anaerobic bread. The specific volume increased with germination time and under aerobic condition, which was positively correlated with

foaming capacity, water absorption and sugar content of flours. After storage for 5 days at room temperature, GBR breads did not change in specific volumes, whereas the control bread significantly reduced in specific volume.

Breads prepared from 4-day and aerobic GBR flour showed a greater specific volume due to their higher sugar contents and foaming capacity. Sugar was produced as a consequence of starch hydrolysis and used as substrates for yeast to produce CO_2 during fermentation. Elgeti et al. (2014) found that the addition of α-amylase or α-glucosidase in quinoa breads resulted in a greater specific volume than the addition of sucrose. The foaming capacity values of GBR flour supported the specific volume results. Kawamura-Konishi, Shoda, Koga, and Honda (2013) reported that the addition of protease from different bacteria improved the specific volume of rice bread as well as the homogeneity of air cells. The dough rheological results also supported the bread specific volume results that GBR dough exhibited a strong dough network and had an increased specific volume (Renzetti & Arendt, 2009).

3.3.2 Crust color

Crust colors were expressed using CIE L^* a^* b^* values with L^* value for lightness, a^* value for redness, and b^* for yellowness. The crust of the control bread showed the highest L^* value, a low a^* value and a high b^* value, indicating a pale and yellowish color. Bread prepared from 4-day aerobically GBR flour had the lowest L^* value, agreeing with its greatest sugar content (Table 2). The L^* value of commercial gluten-free breads was reported to be in the range of 64.71-83.83 (Matos & Rosell, 2012), indicating that GBR breads of the present study was darker, presumably a combination of the increase in reducing sugars and free amino acids, and both greatly enhanced the Maillard reaction. Chareonthaikij et al. (2010) reported that the substitution of wheat flour with GBR flour increased the yellowness of the resultant bread crust.

However, Cornejo and Rosell (2015) found that the increased germination time did not affect the redness and yellowness of bread crust. In the present study, breads prepared from GBR flours exhibited an increase in redness with an exception of 4-day aerobically GBR bread, but a reduction in yellowness, probably due to a loss in pigment from soaking during germination.

3.3.3 Texture analysis

At both day 0 and day 5, the control bread displayed the highest hardness and chewiness, while 4-day aerobic bread showed the lowest ones. Breads prepared from GBR flour showed a decrease in hardness and chewiness due to their higher specific volumes and the hydrolysis of starch and protein. Veluppillai et al. (2010) reported that a replacement of wheat flour with 30% GBR flour in bread displayed a decrease in hardness. After storage for 5 days, hardness and chewiness of all breads significantly increased, assuming predominantly due to starch retrogradation (Eliasson, 1986; Elton, 1969). Nevertheless, 4-day aerobic bread at day 5 had a hardness value similar to the control at day 0, suggesting that effectiveness of GBR in extending shelf-life. Springiness is an indicator for the freshness and elasticity of breads. The control and 2-day GBR breads showed no significant differences among samples on springiness either at day 0 or 5, while 4-day GBR breads had lower springiness because of their wet sticky texture from action of amylases, causing an excessive liquefaction and dextrinization (Cornejo & Rosell, 2015; Hallen, Ibanoglu, & Ainsworth, 2004).

3.3.4 Starch retrogradation

Starch retrogradation or staling is primarily responsible for quality deterioration of bakery products during storage, particularly breads. Breads prepared with GBR flours displayed a significant reduced retrogradation than the control bread (5.41 J/g) (Table 3) at day 0, and

breads prepared from 4-day or aerobically GBR flour had lesser retrogradation than those with 2-day or anaerobically GBR flours, respectively. Breads displayed a significant increase in retrogradation after storage for 5 days, but the same trend as day 0 still followed. The decrease in retrogradation supported the changes in specific volume and texture properties of control and GBR breads and affirmed the impacts of starch degradation on bread quality (Fig. 7). Degraded starch molecules of low and intermediate molecular weights, have been reported to interfere with amylopectin reassociation (Lin & Lineback, 1990). The interactions of starch with sugar and protein could also cause a delay in starch retrogradation (Baek, Yoo, & Lim, 2004; Lin & Lineback, 1990; Rojas, Rosell, & Benedito de Barber, 2001).

3.3.5 Starch molecular size distribution

Starch was isolated from breads and characterized for molecular size distribution to reveal the extent of hydrolysis during the dough preparation and baking (Fig. 12). The differences in Fr. I and II in the control bread relative to those in long-grain rices suggest the occurrence of starch hydrolysis in the control bread from the inherent amylases in the bran, even though it was not germinated. When compared with the profiles of flours (Fig. 9A), the control bread showed the least degradation, whereas breads prepared from GBR flour exhibited more degradation with the greatest found in 4-day aerobically GBR bread. Both Fr. I and Fr. II in all breads significantly decreased, and Fr. III significantly increased, indicating that extensive hydrolysis of starch was achieved in about one hour of fermentation and baking process, and reflecting the amylolytic enzyme activities (Fig. 6). The peak chain lengths of Fr. I and Fr. II in native starch from breads were still similar to those in long-grain rice; however, the peak chain length of Fr. III decreased from DP 1,461 to 279 in 4-day GBR breads. The degradation of molecular size starch supports the decrease in starch retrogradation (Table 3).

When compared with the debranched starch profiles of flours (Fig. 9B) the peak chain lengths of Frs. I, II and III in breads decreased from DP 440 to 150, DP 62 to 43 and DP 27 to 20, respectively. A progressive increase in the proportion of very short chains down to DP 2 was observed in GBR breads, and this increase was enhanced under aerobic condition and at a longer germination time. These short chains would not reassociate and may also interfere with the reassociation of long-chains, thus resulting in less starch retrogradation.

3.4 Statistical analysis

The activities of α-amylase, β-amylase, α-glucosidase, and protease were the key factors that change the properties of flour after germination, thus they were used to analyze the main effects (rice cultivars, germination conditions and duration) and their interactions (Appendices Table I). It was found that rice cultivar, germination condition and duration and their 2-way and 3-way interactions significantly affected the activities of α-amylase, β-amylase and protease ($p < 0.0001$), indicating that it was important to consider not only rice cultivar, germination condition and duration but also their interactions. In contrast, only cultivar ($p < 0.0001$) and germination duration ($p < 0.001$) significantly affected the activity of α-glucosidase, suggesting that germination condition had no effect on the activity of α-glucosidase.

When the specific volume, starch retrogradation and hardness of bread after storage for 5 days from different conditions were analyzed, it was found that germination condition and duration, and most 2-way interactions significantly affected the specific volume, starch retrogradation and hardness of bread after storage for 5 days ($p < 0.0001$), except the 2-way interaction on starch retrogradation (Appendices Table II). The germination condition to produce breads with the greatest specific volume and lowest starch retrogradation and hardness at both day 0 ad 5 was 4-day aerobically germination (Appendices Figure I).

4. Conclusion

The hydrolytic enzymes activated during germination of long-grain and short-grain rices caused significant changes in chemical composition, gelatinization properties, water absorption, foaming capacity, pasting properties and starch molecular size distributions. More changes were observed in long-grain GBRs relative to short-grain GBRs, under aerobic condition and at a longer germination time. However, the extent of starch hydrolysis was influenced not only by activities of amylases, but also by starch composition and fine structure. Gluten-free breads prepared with long-grain GBR exhibited improved specific volume and texture and reduced starch retrogradation. The extensive hydrolysis of starch in breads prepared from 4-day GBR flour resulted in a greater specific volume and reduced hardness, but at the same time decreased springiness and chewiness. Thus, GBR breads can be adapted to specific applications by adjusting the germination conditions. In conclusion, GBR had a potential to be used in gluten-free breads to improve quality and to extent shelf-life.

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6. Tables and Figures

Table 1 Bread formulations

Ingredients (g)	Control Bread	GBR Bread
Brown rice flour	100	-
GBR flour	-	100
Water	80	80
Yeast	3	3
Sugar	7	7
Salt	2	2
Hydroxypropyl methylcellulose	2	2

Table 2 Chemical composition, gelatinization properties, and functional properties of GBR flour of long- and short-grain rices.[1]

	Chemical composition (%, db)					Gelatinization properties					Functional properties	
	Protein	Lipid	Ash	Apparent amylose	Sugar	T_o (°C)	T_p (°C)	T_e (°C)	ΔT (°C)	Enthalpy (J/g)	Water absorption (g/g)	Foaming capacity (%, db)
Long-grain rice												
Brown rice (control)	8.43^{e} ± 0.00	2.72^{a} ± 0.01	1.52^{a} ± 0.00	23.24^{a} ± 0.09	0.34^{e} ± 0.00	73.54^{e} ± 0.11	78.46^{e} ± 0.08	83.90^{d} ± 0.05	10.36^{ab} ± 0.15	8.42^{a} ± 0.05	1.04^{e} ± 0.05	0.12^{e} ± 0.01
2-day aerobic	8.74^{c} ± 0.00	2.65^{b} ± 0.02	1.35^{b} ± 0.01	22.00^{c} ± 0.10	0.91^{c} ± 0.01	74.90^{c} ± 0.17	79.16^{d} ± 0.05	83.93^{d} ± 0.15	9.03^{c} ± 0.26	7.63^{c} ± 0.08	1.98^{b} ± 0.00	0.19^{c} ± 0.00
2-day anaerobic	8.60^{d} ± 0.00	2.67^{b} ± 0.02	1.29^{d} ± 0.00	22.59^{b} ± 0.13	0.61^{d} ± 0.02	74.43^{d} ± 0.08	79.35^{c} ± 0.08	85.10^{b} ± 0.10	10.67^{a} ± 0.03	8.29^{b} ± 0.05	2.18^{a} ± 0.01	0.16^{d} ± 0.00
4-day aerobic	8.97^{a} ± 0.01	2.13^{d} ± 0.02	1.33^{c} ± 0.01	21.02^{e} ± 0.07	4.29^{a} ± 0.01	76.07^{a} ± 0.07	80.72^{a} ± 0.09	86.25^{a} ± 0.20	10.18^{b} ± 0.25	7.40^{e} ± 0.02	1.67^{d} ± 0.01	0.29^{a} ± 0.00
4-day anaerobic	8.85^{b} ± 0.00	2.19^{c} ± 0.01	1.26^{e} ± 0.00	21.38^{d} ± 0.06	1.05^{b} ± 0.01	75.59^{b} ± 0.07	79.75^{b} ± 0.15	84.68^{c} ± 0.06	9.09^{c} ± 0.02	7.52^{d} ± 0.05	1.86^{c} ± 0.01	0.24^{b} ± 0.00
Short-grain rice												
Brown rice (control)	8.87^{d} ± 0.04	2.82^{a} ± 0.00	1.64^{a} ± 0.00	12.55^{a} ± 0.26	0.60^{e} ± 0.01	67.97^{b} ± 0.08	74.55^{c} ± 0.12	82.01^{c} ± 0.15	14.04^{a} ± 0.17	9.22^{ab} ± 0.25	1.40^{e} ± 0.00	0.06^{e} ± 0.01
2-day aerobic	9.10^{b} ± 0.01	2.75^{b} ± 0.00	1.29^{b} ± 0.00	11.30^{c} ± 0.05	2.03^{c} ± 0.02	71.46^{a} ± 0.06	76.99^{ab} ± 0.12	84.50^{a} ± 0.14	13.05^{b} ± 0.20	8.85^{b} ± 0.23	1.61^{b} ± 0.01	0.09^{c} ± 0.00
2-day anaerobic	8.99^{c} ± 0.02	2.76^{b} ± 0.00	1.16^{d} ± 0.00	11.67^{b} ± 0.04	1.13^{d} ± 0.01	68.36^{b} ± 0.43	74.16^{c} ± 0.20	82.10^{c} ± 0.39	13.74^{a} ± 0.38	9.41^{a} ± 0.33	1.63^{a} ± 0.01	0.07^{d} ± 0.00
4-day aerobic	9.30^{a} ± 0.02	2.61^{d} ± 0.00	1.22^{c} ± 0.00	10.13^{d} ± 0.16	8.63^{a} ± 0.19	71.64^{a} ± 0.25	77.22^{a} ± 0.35	83.26^{b} ± 0.40	11.62^{c} ± 0.40	9.57^{a} ± 0.12	1.45^{d} ± 0.01	0.10^{a} ± 0.00
4-day anaerobic	9.15^{b} ± 0.01	2.70^{c} ± 0.00	0.99^{e} ± 0.00	11.29^{c} ± 0.06	2.93^{b} ± 0.02	71.17^{a} ± 0.45	76.71^{ab} ± 0.38	83.16^{b} ± 0.71	11.98^{c} ± 0.46	8.17^{c} ± 0.30	1.53^{c} ± 0.01	0.10^{b} ± 0.01

[1]Mean values of two measurements in the same column for the same rice cultivar with different letters are significantly different ($p<0.05$).

Table 3 Characteristics of breads prepared from long-grain rice of different germination conditions at day 0 and day 5.[1]

Breads	Specific volume (mL^3/g)	Textural Attributes			Retrogradation (J/g)	Crust Color		
		Hardness (N)	Springiness	Chewiness (N)		L^*	a^*	b^*
At day 0								
Brown rice (control)	$1.59^{d} \pm 0.01$	$39.7^{a} \pm 1.0$	$0.92^{a} \pm 0.04$	$23.6^{a} \pm 2.2$	$5.41^{a} \pm 0.15$	$64.50^{a} \pm 2.17$	$7.98^{d} \pm 0.13$	$25.49^{a} \pm 0.47$
2-day aerobic	$1.65^{c} \pm 0.02$	$16.7^{c} \pm 0.2$	$0.84^{a} \pm 0.05$	$10.5^{c} \pm 0.9$	$1.24^{c} \pm 0.04$	$51.03^{c} \pm 1.55$	$12.88^{a} \pm 0.93$	$18.32^{c} \pm 1.89$
2-day anaerobic	$1.57^{e} \pm 0.01$	$24.5^{b} \pm 1.5$	$0.90^{a} \pm 0.05$	$16.6^{b} \pm 3.1$	$1.44^{b} \pm 0.01$	$55.58^{b} \pm 1.35$	$11.90^{b} \pm 0.74$	$23.45^{b} \pm 0.92$
4-day aerobic	$1.75^{a} \pm 0.01$	$4.3^{e} \pm 0.8$	$0.62^{b} \pm 0.06$	$1.1^{d} \pm 0.1$	$0.60^{e} \pm 0.01$	$40.52^{e} \pm 0.56$	$4.75^{e} \pm 0.80$	$3.58^{e} \pm 0.45$
4-day anaerobic	$1.72^{b} \pm 0.00$	$8.6^{d} \pm 1.3$	$0.62^{b} \pm 0.05$	$2.2^{d} \pm 0.3$	$1.03^{d} \pm 0.01$	$43.51^{d} \pm 0.29$	$9.48^{c} \pm 0.28$	$8.67^{d} \pm 0.48$
At day 5								
Brown rice (control)	$1.49^{e} \pm 0.00$	$179.7^{a} \pm 5.0$	$0.78^{a} \pm 0.05$	$36.5^{a} \pm 1.3$	$6.70^{a} \pm 0.37$			
2-day aerobic	$1.66^{c} \pm 0.02$	$145.1^{c} \pm 4.8$	$0.79^{a} \pm 0.03$	$24.8^{c} \pm 0.8$	$5.12^{c} \pm 0.07$			
2-day anaerobic	$1.58^{d} \pm 0.00$	$155.9^{b} \pm 5.7$	$0.78^{a} \pm 0.01$	$28.4^{b} \pm 1.3$	$6.12^{b} \pm 0.06$			
4-day aerobic	$1.75^{a} \pm 0.00$	$43.5^{e} \pm 3.3$	$0.66^{b} \pm 0.02$	$8.2^{e} \pm 0.5$	$3.98^{e} \pm 0.05$			
4-day anaerobic	$1.72^{b} \pm 0.00$	$72.9^{d} \pm 2.1$	$0.66^{b} \pm 0.03$	$15.9^{d} \pm 0.9$	$4.90^{d} \pm 0.06$			

[1]Mean values of two measurements in the same column within each day with different letters are significantly different ($p<0.05$).

Table 4 Molecular weight distribution (%) of native and debranched starch from long- and short-grain rices and breads by HPSEC.[1]

	Native starch			Debranched starch		
	Fr. I	Fr. II	Fr. III	Fr. I	Fr. II	Fr. III
Long-grain rice						
Brown rice	51.1^{a} ± 0.1	16.4^{b} ± 0.6	32.5^{d} ± 0.6	11.0^{a} ± 1.3	24.1^{a} ± 0.1	65.0^{a} ± 2.3
2-day aerobic	30.3^{c} ± 0.1	25.4^{a} ± 0.2	44.4^{c} ± 0.4	12.2^{a} ± 0.4	24.1^{a} ± 1.8	63.7^{a} ± 1.3
2-day anaerobic	37.8^{b} ± 1.9	16.1^{b} ± 1.1	46.1^{b} ± 0.8	12.2^{a} ± 0.2	24.0^{a} ± 2.5	63.8^{a} ± 2.8
4-day aerobic	18.5^{d} ± 0.5	25.6^{a} ± 0.4	55.9^{a} ± 0.2	12.0^{a} ± 0.7	23.7^{a} ± 0.7	64.3^{a} ± 0.0
4-day anaerobic	19.1^{d} ± 0.1	25.6^{a} ± 0.1	55.4^{a} ± 0.0	12.0^{a} ± 0.4	24.8^{a} ± 0.0	63.3^{a} ± 0.4
Short-grain rice						
Brown rice	55.3^{a} ± 0.1	9.5^{e} ± 0.0	35.2^{d} ± 0.1	2.9^{d} ± 0.1	29.8^{a} ± 0.5	67.3^{b} ± 0.6
2-day aerobic	44.5^{c} ± 1.2	13.4^{b} ± 0.1	42.2^{b} ± 1.1	3.8^{c} ± 0.1	27.2^{b} ± 0.1	69.0^{a} ± 0.2
2-day anaerobic	53.6^{a} ± 0.7	17.9^{a} ± 0.0	28.5^{e} ± 0.7	3.7^{c} ± 0.4	27.2^{b} ± 0.1	69.2^{a} ± 0.4
4-day aerobic	41.9^{d} ± 0.8	13.1^{d} ± 0.1	45.1^{a} ± 0.8	9.6^{a} ± 0.1	26.4^{c} ± 0.2	64.0^{d} ± 0.1
4-day anaerobic	48.4^{b} ± 0.5	13.2^{c} ± 0.0	38.4^{c} ± 0.5	8.0^{b} ± 0.1	26.9^{bc} ± 0.1	65.1^{c} ± 0.0
Breads						
Brown rice	34.2^{a} ± 1.3	32.9^{a} ± 0.5	32.9^{e} ± 0.8	20.5^{a} ± 0.1	19.3^{ab} ± 0.2	60.2^{e} ± 0.1
2-day aerobic	11.3^{c} ± 0.4	29.3^{c} ± 0.2	59.4^{c} ± 0.6	11.7^{d} ± 0.0	17.8^{c} ± 0.0	70.5^{b} ± 0.0
2-day anaerobic	15.1^{b} ± 1.6	31.5^{b} ± 0.1	53.4^{d} ± 1.6	15.8^{b} ± 0.1	20.1^{a} ± 0.2	64.2^{d} ± 0.3
4-day aerobic	7.6^{d} ± 0.1	21.3^{e} ± 0.9	71.1^{a} ± 0.7	12.8^{c} ± 0.4	14.8^{d} ± 0.3	72.5^{a} ± 0.1
4-day anaerobic	9.1^{cd} ± 0.2	25.2^{d} ± 0.4	65.7^{b} ± 0.1	11.1^{e} ± 0.1	19.2^{b} ± 0.5	69.7^{c} ± 0.4

[1]Mean values of two measurements in the same column within rice cultivars and breads with different letters are significantly different ($p<0.05$).

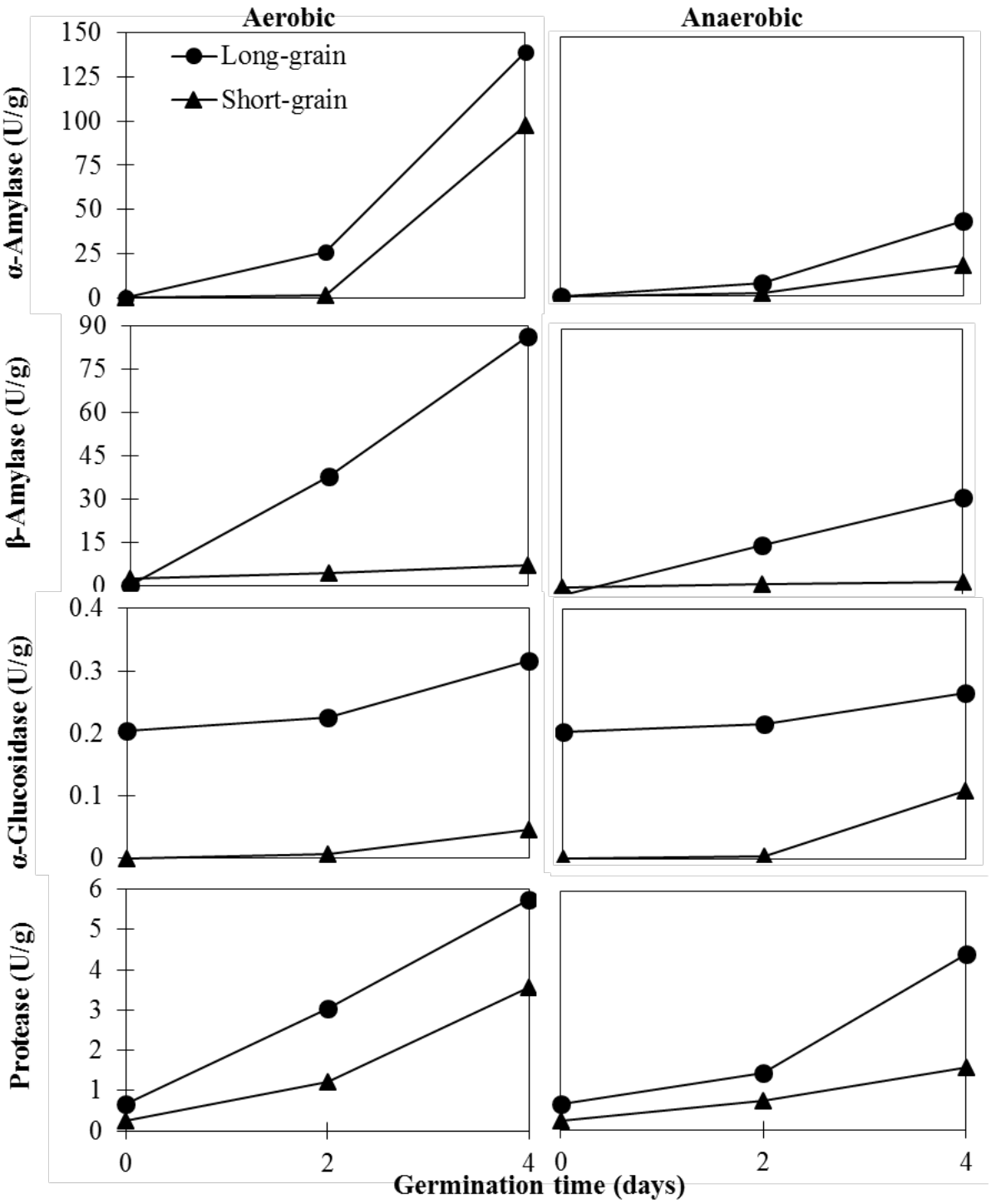

Figure 6 Activities of α-amylase, β-amylase, α-glucosidase and protease in GBR of different cultivars and germination conditions.

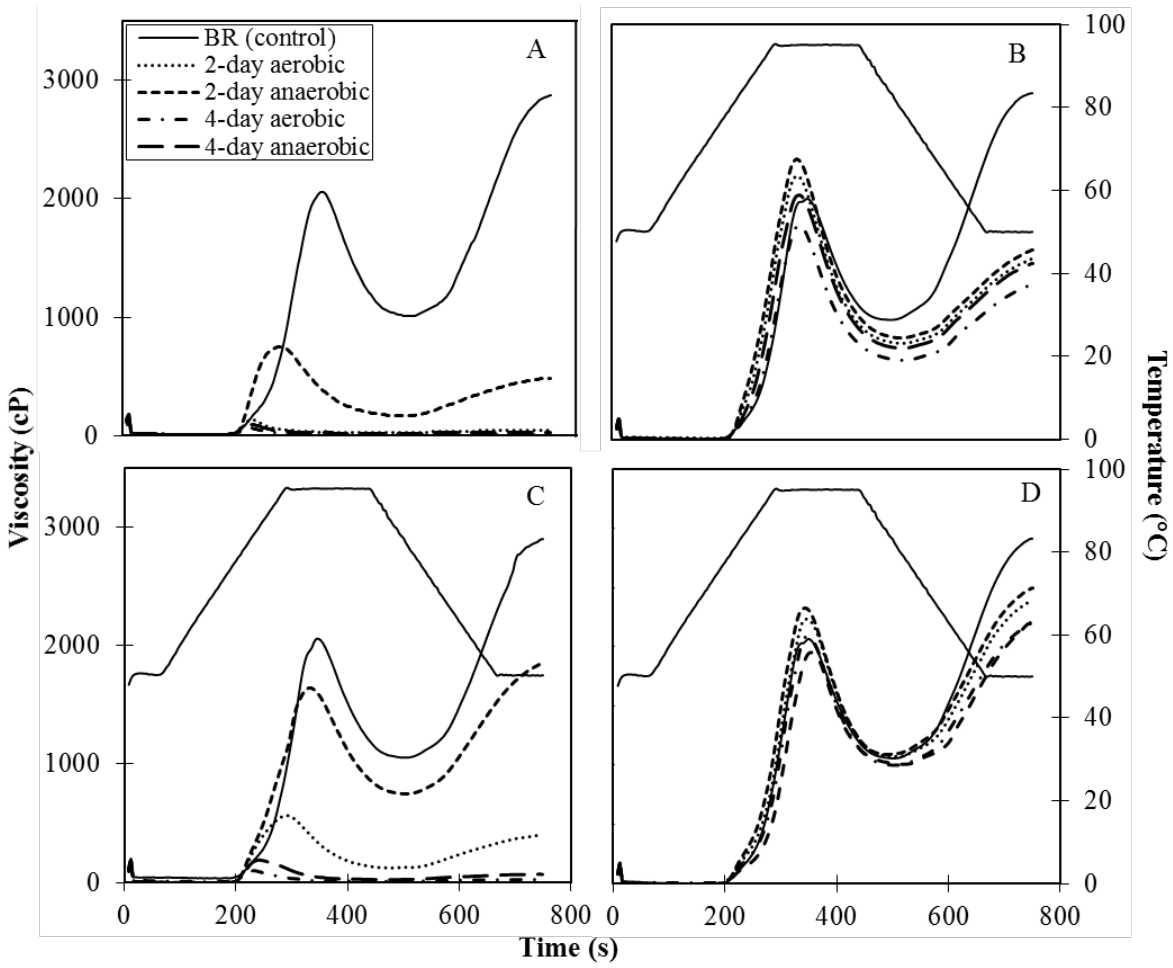

Figure 7 Pasting profiles of long-grain brown rice and GBR from different germination conditions using water (A), 0.50 mM $AgNO_3$ (B), 0.1% N-ethylmaleimide (C), and a mixture (50:50) of 1.0 mM $AgNO_3$ and 0.2% N-ethylmaleimide (D).

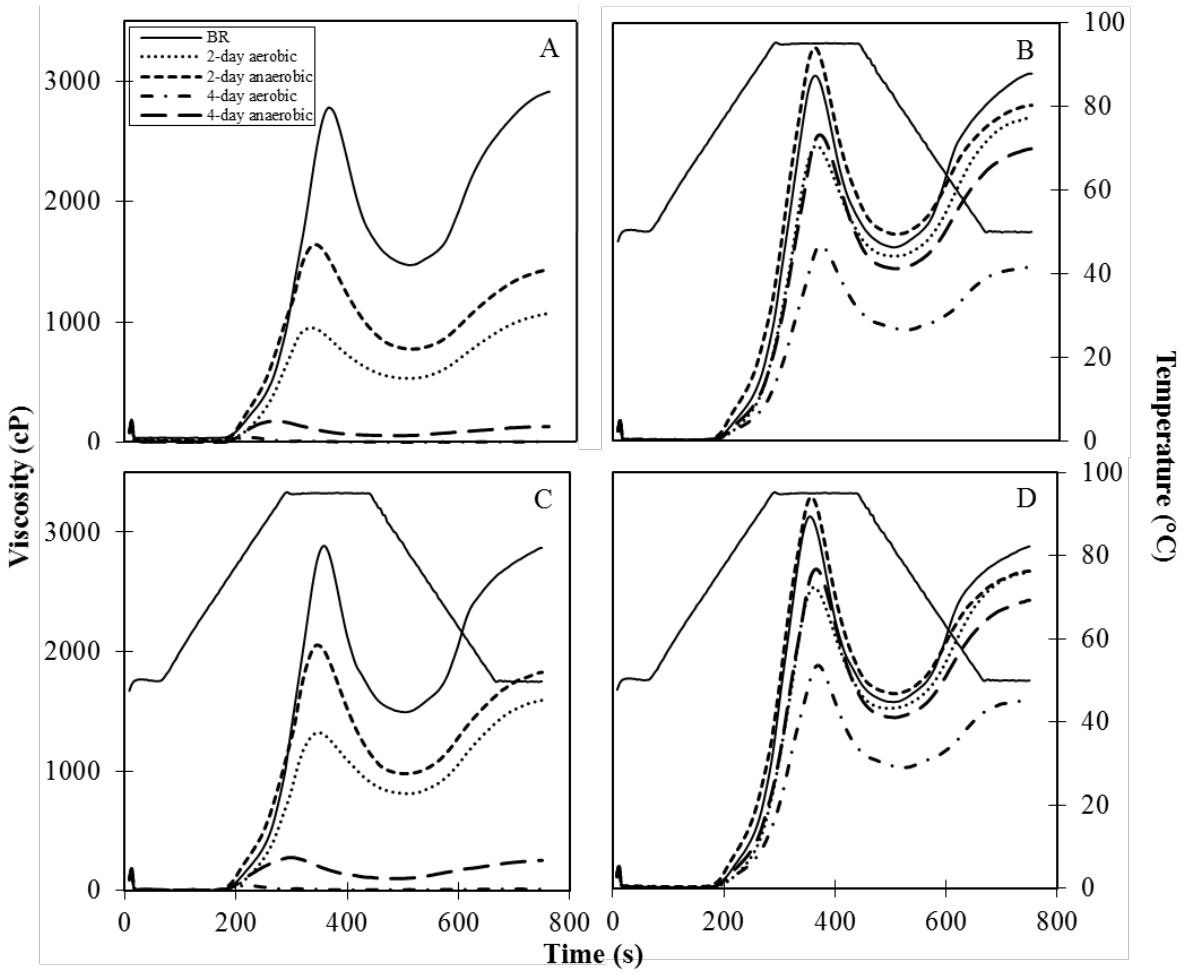

Figure 8 Pasting profiles of short-grain brown rice and GBR from different germination conditions using water (A), 0.50 mM $AgNO_3$ (B), 0.1% N-ethylmaleimide (C), and a mixture (50:50) of 1.0 mM $AgNO_3$ and 0.2% N-ethylmaleimide (D).

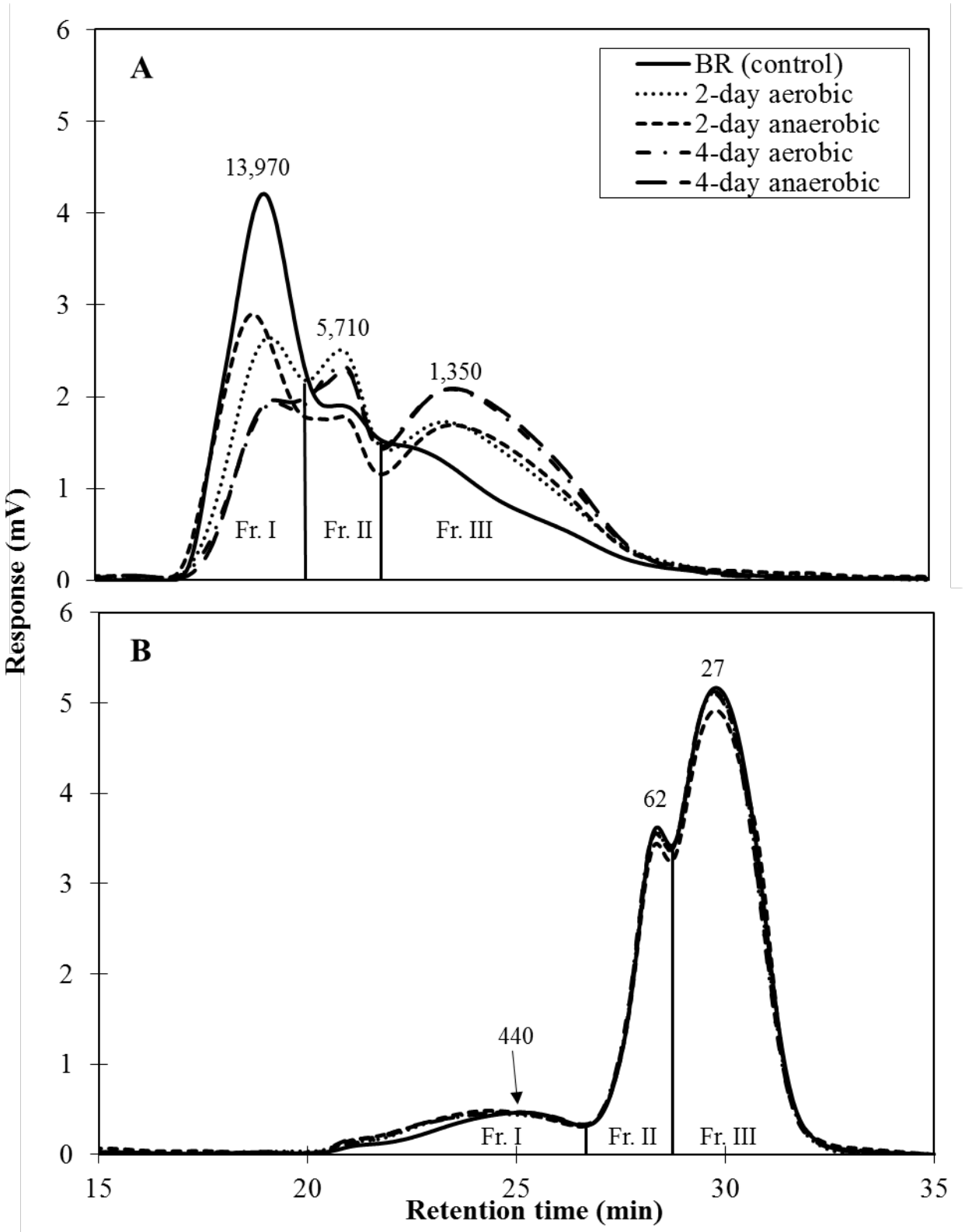

Figure 9 Normalized high-performance size-exclusion chromatograms of native (A) and debranched starch (B) from germinated long-grain rice under different germination conditions**.**

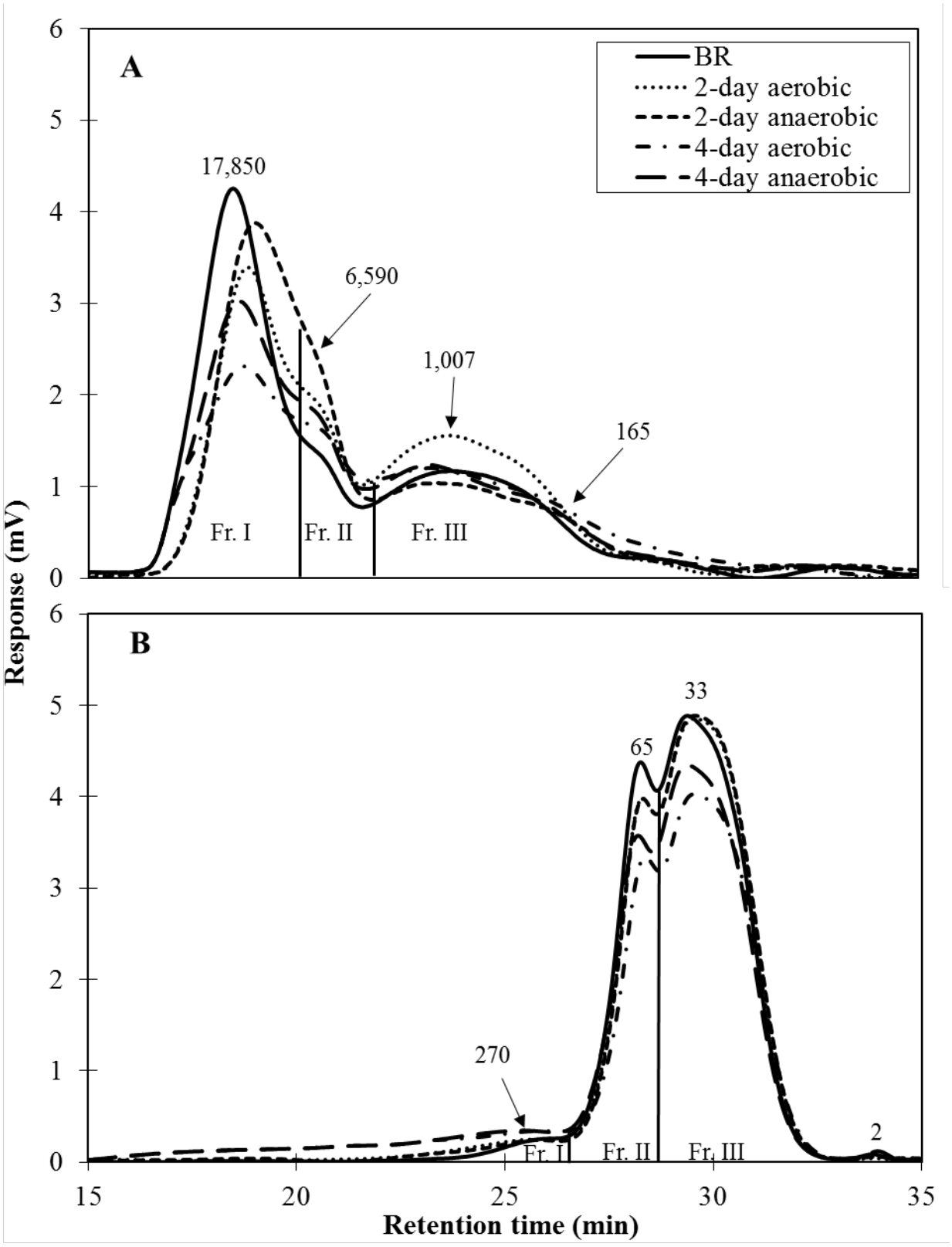

Figure 10 Normalized high-performance size-exclusion chromatograms of native (A) and debranched starch (B) from germinated short-grain rice under different germination conditions.

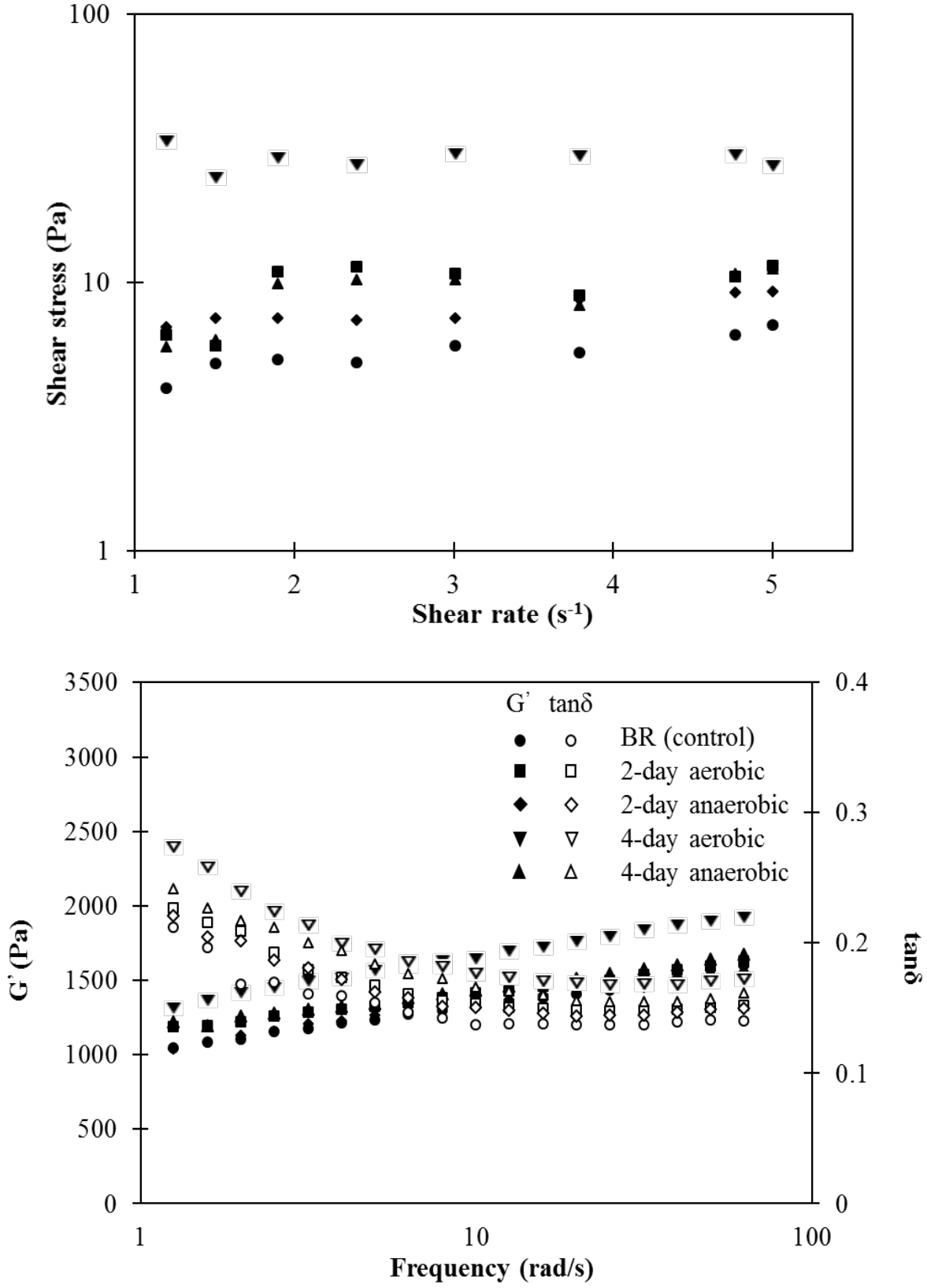

Figure 11 Rheology and flow behavior of dough prepared from brown rice under different germination conditions.

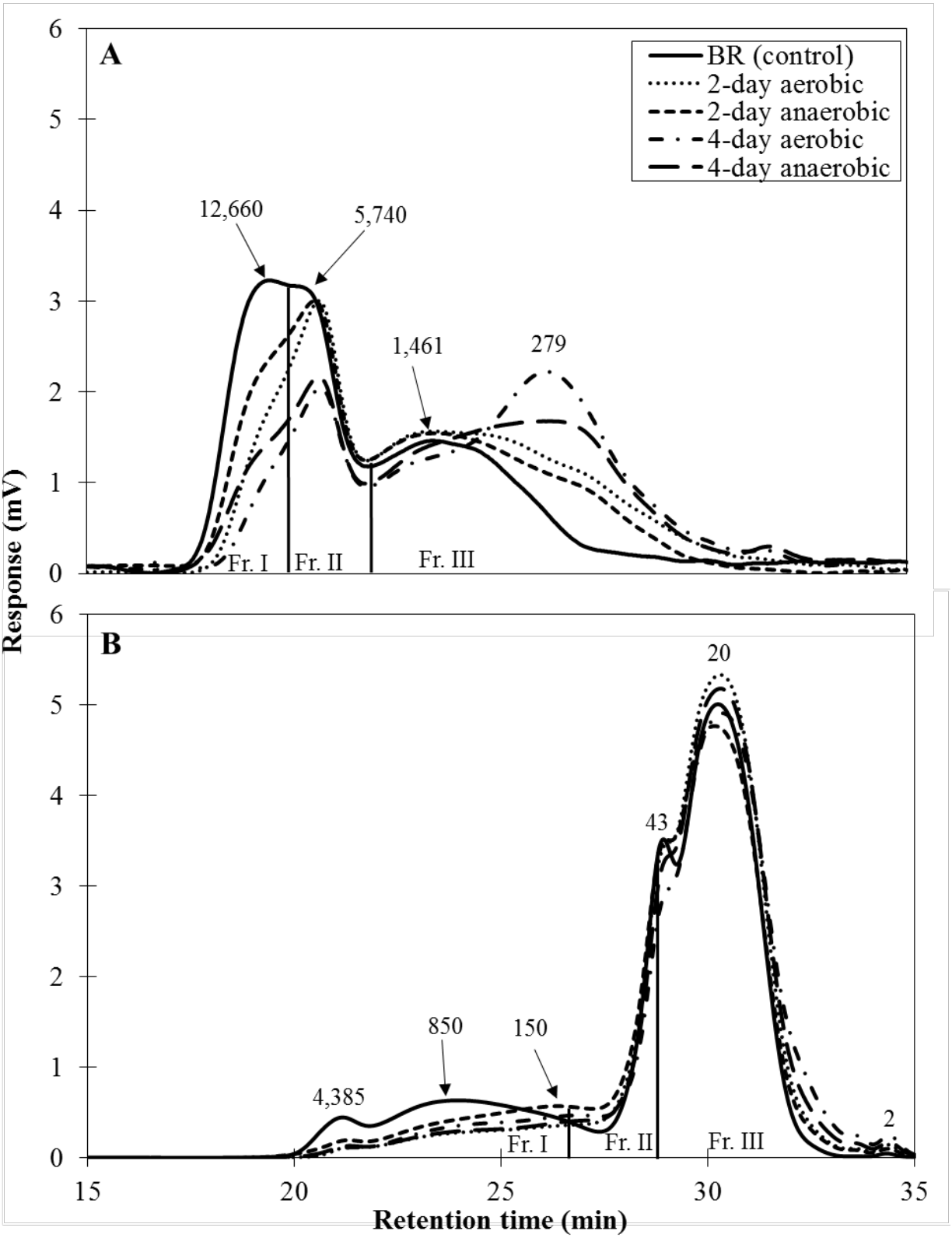

Figure 12 Normalized high-performance size-exclusion chromatography (HPSEC) of native (A) and debranched starch (B) from breads prepared from germinated long-grain brown rice under different germination conditions.

IV. Conclusion

Flour properties were mainly affected by hydrolytic enzymes activated during germination, causing changes in chemical composition, physiochemical properties and starch distributions. Long-grain GBRs displayed greater changes than those of short-grain rices with more pronounced in aerobic condition and longer time germination. However, short-grain GBR exhibited more degraded starch than long-grain GBR despite of their lower enzyme activities. Germination improved the quality of gluten-free breads prepared from long-grain rices on specific volume, texture and starch retrogradation. Different germination conditions produced different bread qualities. Thus, it is important to determine the germination conditions that would produce breads with best qualities. In conclusion, GBR had a potential to be used in gluten-free breads with better quality and longer-shelf-life.

1. Appendices

Table I Analysis of variance (ANOVA) results for the activities of α-amylase, β-amylase, α-glucosidase and protease

Source	DF	*F*-Ratio	*p*-Value
α-amylase			
Rice cultivar (X_1)	1	85,032.02	<.0001
Germination condition (X_2)	1	329,985.20	<.0001
Germination duration (X_3)	1	600,393.50	<.0001
X_1X_2	1	10,925.01	<.0001
X_1X_3	1	11,981.25	<.0001
X_2X_3	1	219,320.20	<.0001
$X_1X_2X_3$	1	99.91	<.0001
β-amylase			
Rice cultivar (X_1)	1	72,463.38	<.0001
Germination condition (X_2)	1	18,279.79	<.0001
Germination duration (X_3)	1	13,968.52	<.0001
X_1X_2	1	15,216.10	<.0001
X_1X_3	1	11,308.05	<.0001
X_2X_3	1	3,582.48	<.0001
$X_1X_2X_3$	1	2,794.81	<.0001
α-glucosidase			
Rice cultivar (X_1)	1	140.69	<.0001
Germination condition (X_2)	1	0.00	0.9733
Germination duration (X_3)	1	15.51	0.1156
X_1X_2	1	2.67	0.0006
X_1X_3	1	0.01	0.9336
X_2X_3	1	0.13	0.7250
$X_1X_2X_3$	1	2.11	0.1596

Table I (Cont.)

Source	DF	*F*-Ratio	*p*-Value
Protease			
Rice cultivar (X_1)	1	4,159.35	<.0001
Germination condition (X_2)	1	2,131.89	<.0001
Germination duration (X_3)	1	5,795.02	0.0008
X_1X_2	1	14.83	<.0001
X_1X_3	1	483.00	<.0001
X_2X_3	1	108.68	<.0001
$X_1X_2X_3$	1	237.28	<.0001

Table II Analysis of variance (ANOVA) results for the specific volume, starch retrogradation and hardness of breads at day 5

Source	DF	*F*-Ratio	*p*-Value
Specific volume			
Germination condition (X_1)	1	95.79	<.0001
Germination duration (X_2)	1	353.34	<.0001
X_1X_2	1	16.33	0.002
Starch retrogradation			
Germination condition (X_1)	1	988.39	<.0001
Germination duration (X_2)	1	1,502.08	<.0001
X_1X_2	1	1.85	0.1986
Hardness			
Germination condition (X_1)	1	91.40	<.0001
Germination duration (X_2)	1	1,939.09	<.0001
X_1X_2	1	19.76	0.001

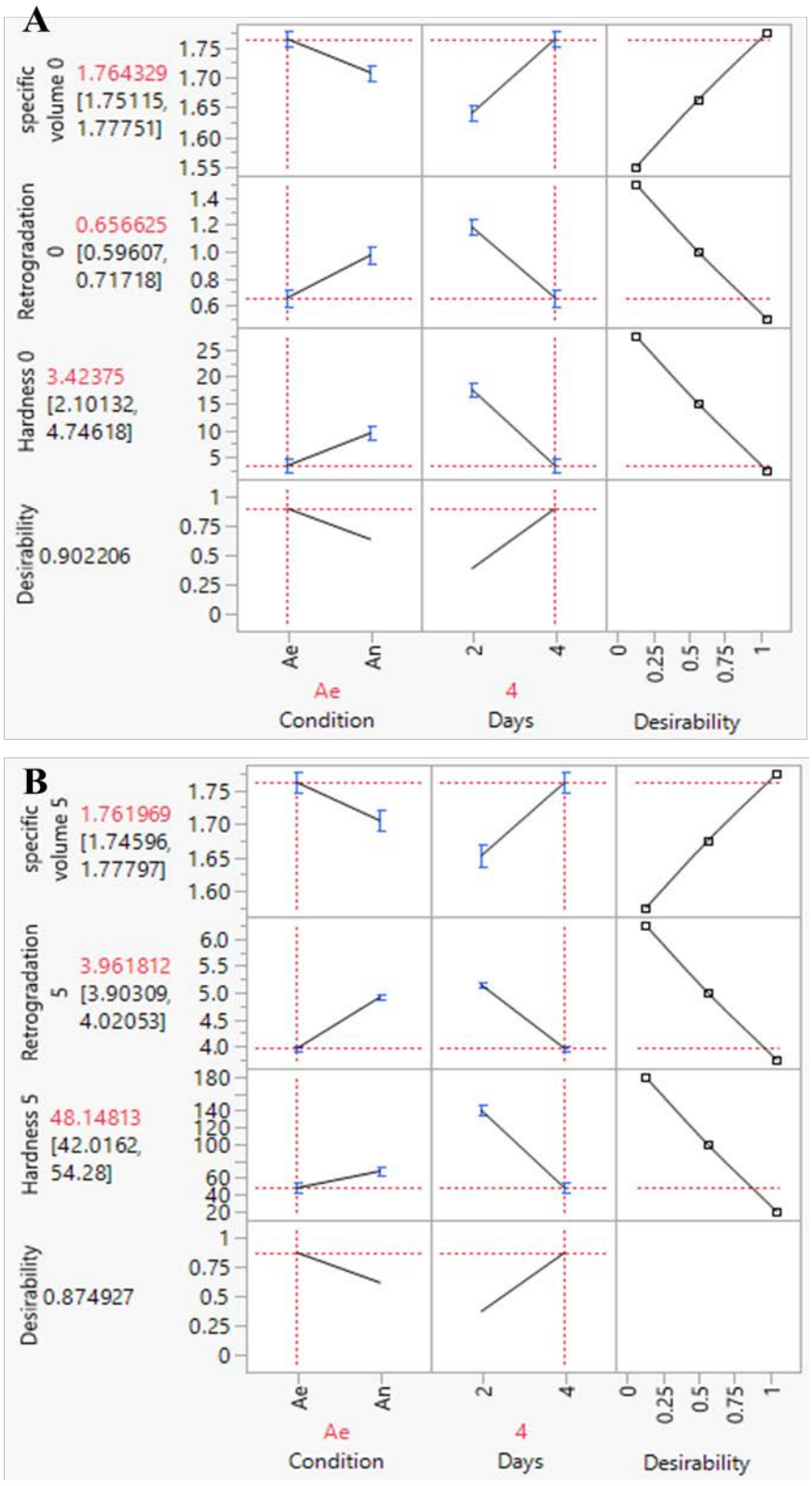

Figure I Prediction profiler of maximum specific volume and minimize starch retrogradation and hardness of breads at day 0 (A) and 5 (B).